CANINE CARDIAC CALCIUM-DEPENDENT PROTEASES: RESOLUTION OF TWO FORMS WITH DIFFERENT REQUIREMENTS FOR CALCIUM

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1. Introduction

The presence of a Ca^{2+}-dependent protease in the soluble fraction of rat brain extracts which has optimum activity at neutral pH was reported in [1]. Despite the fact that protease activities closely related to the rat brain enzyme have been detected in many mammalian tissues [2,3] and purified from porcine [4] and chicken [5] skeletal muscle, little is known about the function of these proteases in the cell. Such protease activities may be involved in regulation of muscle fiber turnover since they disrupt the myofibril Z-line [4,6]. Ca^{2+}-dependent protease activities which degrade filamin–actin complexes [7] and microtubule-associated assembly factors [8] have also been described. Thus, Ca^{2+}-dependent proteases may be responsible for initiating turnover of a variety of cellular ultrastructures.

One of the major problems associated with assessing the physiological significance of these protease activities in the cell is that they require a much higher concentration of free-Ca^{2+} than is normally present in muscle cells [1,4]. In the following studies, two Ca^{2+}-dependent protease activities from canine cardiac extracts were separated and partially purified. Although many properties of the two activities were similar, one of the forms was found to be active at free-Ca^{2+} concentrations which may be attained in muscle cells during contraction.

2. Experimental

2.1. *Protease assay*

Protease activity was determined using a modification of the assay in [10] which is based on the reaction of fluorescamine with primary amine groups produced by proteolytic cleavage of protein substrates. The method used here was that in [11] except that activity was determined at 20°C rather than 30°C since the enzyme was more stable at the lower temperature, and all protease assays except where noted were done in the presence of 5 mM $CaCl_2$, at pH 6.8. A unit of protease activity is defined as the amount of enzyme which produces one micromole of glycine-equivalent free amine from succinylated bovine serum albumin per minute at 20°C and pH 6.8.

2.2. *Preparation of canine cardiac proteases*

Hearts were excised from mongrel dogs anesthetized with secobarbital. The hearts were allowed to beat in ice-cold isotonic saline solution for a few minutes to expel excess blood. They were then immediately placed in a −70°C freezer and stored for up to several months before being used. All purification steps unless otherwise noted were performed at 5°C. The hearts were thawed and trimmed of fat, minced in a commercial meat grinder and mixed with 2 vol. 50 mM imidazole–HCl, 5 mM EDTA, 42 mM mercaptoethanol (pH 7.4) (buffer A) containing in addition 0.25 M sucrose. The mixture was blended in a Waring blender using two 10 s bursts at low speed. The homogenate was centrifuged at 14 000 × *g* for 20 min, and the supernatant was mixed with 500 ml DEAE–Sepharose CL-6B equilibrated with buffer A. The DEAE–Sepharose was poured into a 5.0 cm diam. column, and the gel was washed with 800 ml buffer A containing 0.1 M NaCl. The protease activity was then eluted with buffer A containing 0.4 M NaCl. Cold, neutralized, saturated ammonium sulfate (1.5 vol.) was added to the pooled protease fractions. After 20 min at 0°C, the precipitate was collected by

centrifugation at 10 000 × *g* for 20 min. The resulting precipitate was resuspended in buffer A to 20 ml final vol. and dialyzed against 1 l buffer A for 60 h. The insoluble material in the dialyzed preparation was removed by centrifugation, and the preparation was applied to a 2.6 × 86 cm column of Ultrogel AcA 34 equilibrated in buffer A. The pooled column fractions containing protease activity from Ultrogel chromatography were applied to a 1.6 × 12 cm column of DEAE–Sephadex A-25 equilibrated in 50 mM imidazole–HCl, 5 mM EDTA, 1 mM dithiothreitol (pH 7.4) (buffer B), and the column was washed with 90 ml buffer B. The protease activities were eluted with a 200 ml 0–0.5 M NaCl gradient in buffer B. The protease peaks from this step were pooled separately and dialyzed overnight against buffer B containing 50% (v/v) glycerol and stored at −20°C.

2.3. *EDTA-free protease samples*

Prior to determining the Ca^{2+} requirements of the protease, samples of peak I and peak II protease were exchanged for EDTA-free buffer B containing 0.2 M NaCl by gel filtration on Sephadex G-50, medium mesh. The rapid method of [12] was used. The sample size was 0.2 ml, and the Sephadex G-50 wet gel vol. 2.0 ml. >70% of the activity of both proteases was recovered. It was estimated that <1% of the EDTA remained in the sample after filtration, based on retention of [^{32}P]ATP in the gel matrix using the same gel bed volume and sample size (D. Brown, unpublished observation).

2.4. *Other methods*

Protein concentrations for monitoring protease purification were determined by the modification [13] of the method in [14]. Proteins were detected in chromatography fractions by the rapid fluorometric method in [15].

2.5. *Materials*

Grade II imidazole was obtained from Sigma Chemical Co. and was recrystallized from acetone before being used in buffers. DEAE–Sepharose CL-6B, DEAE–Sephadex A-25 and Sephadex G-50 were products of Pharmacia Fine Chemicals obtained through Sigma. Ultrogel AcA 34 was purchased from LKB. Bovine serum albumin (fraction V, fatty acid-free), leupeptin, soybean trypsin inhibitor, and protamine (grade IV, free base) were obtained from Sigma. Fluorescamine was purchased from Aldrich Chemical Co. All other chemicals used were of reagent grade.

Table 1
Purification of dog heart calcium-dependent proteases

Procedure	Protease activity (units)	Protein (mg)	Purification	Recovery (% yield)
14 000 × *g* supernatant	(224)[a]	17 098	(1)[a]	(100)[a]
DEAE–Sepharose	224	1680	10.2	100
Ammonium sulfate ppt.	185	988	14.3	83
Ultrogel AcA 34	125	201	47.3	56
DEAE–Sephadex peak I	14.6	7.65	(146)[b]	(6.5)[b]
DEAE–Sephadex peak II	98	5.77	(1298)[b]	(44)[b]

[a] Based on activity recovered from DEAE–Sepharose chromatography, since there was a protease inhibitor in the extract (see text)
[b] Based on total protease activity in the DEAE–Sepharose fraction

The calcium-dependent proteases were partially purified from 510 g dog heart by the procedure in section 2

3. Results

3.1. *Purification of proteases*

Two fractions of canine cardiac Ca^{2+}-dependent protease were partially purified utilizing the purification scheme described above (table 1). Peak I protease eluted from DEAE–Sephadex at 60 mM NaCl (fig.1) and was purified 145-fold on the basis of total protease activity. Peak II protease eluted at 160 mM NaCl (fig.1), and was purified 1298-fold. The total protease activity in the 14 000 × *g* supernatant was estimated from the protease activity recovered from DEAE–Sepharose chromatography. This estimation is necessary since canine cardiac extracts contained a potent inhibitory activity presumably related to the factors described in rabbit cardiac [16] and bovine cardiac [17] extracts which made it impossible to detect protease activity.

3.2. *Molecular weights of proteases*

The molecular weights of the isolated proteases were estimated by analytical gel filtration on an Ultrogel AcA 34 column. As shown in fig.2, panel A, the individual proteases were indistinguishable on the basis of their gel filtration profiles. The apparent molecular weight of the protease forms was 135 000 (fig.2, panel B).

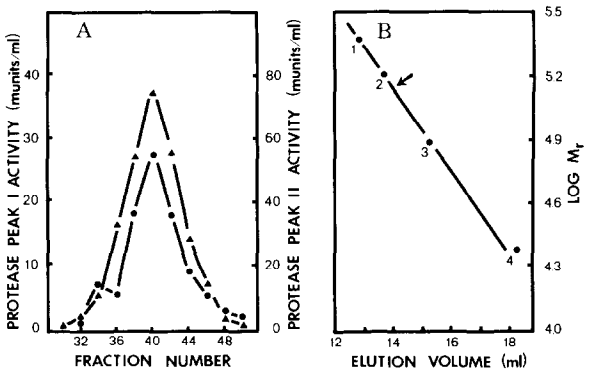

Fig.2. Gel filtration of peak I and peak II proteases. Protease samples were subjected to gel filtration on a calibrated 1.0 × 26 cm Ultrogel AcA 34 column equilibrated in 50 mM imidazole–HCl, 5 mM EDTA, 200 mM NaCl, 42 mM mercaptoethanol (pH 7.4). Peak I and peak II proteases were applied at 1.3 units/ml and 3.4 units/ml, respectively. Samples (10 μl) of column fractions were assayed for protease activity using succinylated bovine serum albumin as a substrate. (A) Gel filtration profile of peak I protease (•) and peak II protease (▲). (B) Estimated molecular weight of the protease forms. The calibration standards used were: (1) catalase; (2) aldolase; (3) lactoperoxidase; (4) chymotrypsinogen A. The arrow marks the elution volume of the proteases.

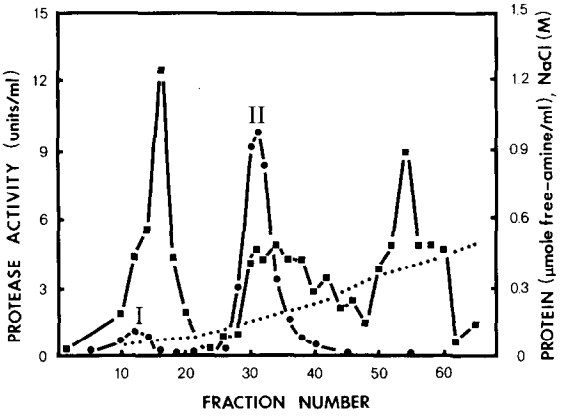

Fig.1. Separation of dog heart peak I and peak II proteases on DEAE–Sephadex A-25 chromatography. Pooled protease fractions from the Ultrogel AcA 34 gel filtration step (table 1) were applied to a DEAE–Sephadex A-25 column and chromatography performed as in section 2. Column fractions were assayed for protease activity (•) using succinylated bovine serum albumin as substrate. Relative protein concentrations in column fractions (▪) were determined as in [15], and are expressed as glycine-equivalent free amine concentration in the fractions. The chloride concentrations (· · · ·) of column fractions were determined with a Corning Model 920 M chloridometer.

3.3. *pH optima of proteases*

Both proteases forms had maximum activity at pH 7.0 using succinylated protamine as substrate (not shown). Peak I protease had a broader pH profile and slightly more activity at pH 5.5–7.0 than peak II protease. Similar results were reported for peak I and peak II proteases from rabbit skeletal muscle [11].

3.4. *Effect of protease inhibitors*

In order to further characterize the two protease forms, the effect of various protease inhibitors on the two activities was studied. At 5 mM, EDTA, *N*-ethylmaleimide and iodoacetamide completely inhibited both protease forms. At 20 μM, leupeptin also totally inactivated both forms. In a separate experiment, it was shown that peak I and peak II proteases were inhibited 50% in the presence of 1.6 μM leupeptin and 2.5 μM leupeptin, respectively. Phenylmethylsulfonyl fluoride at 1 mM inhibited peak I protease 45% and peak II protease 52%, while soybean trypsin

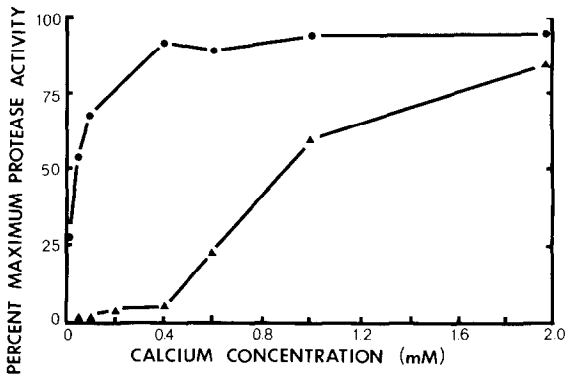

Fig.3. Effect of calcium on EDTA-free dog heart proteases. Samples of peak I and peak II protease were exchanged for EDTA-free buffer as in section 2. Samples (10 μl) of peak I protease (•) or peak II protease (▲), at 0.4 units/ml and 1.2 units/ml, respectively, were assayed for protease activity using succinylated bovine serum albumin substrate containing various concentrations of calcium.

inhibitor at 0.1 mg/ml did not significantly inhibit either protease form. Thus, both protease forms appear to be divalent-cation requiring proteases which are inhibited by sulfhydryl modifying reagents. Similar properties have been described for Ca^{2+}-dependent proteases from rabbit skeletal muscle [4] and human platelets [18].

3.5. *Activation by Ca^{2+}*

Both protease forms were activated by the addition of Ca^{2+} (fig.3). The $A_{0.5}$ of peak I protease for Ca^{2+} was ~40 μM. The activation of peak II protease yielded a sigmoidal saturation curve similar to curves described for a skeletal muscle Ca^{2+}-dependent protease [4,7]. There was very little activity until Ca^{2+} was >400 μM. The $A_{0.5}$ of peak II protease for Ca^{2+} was 800 μM. Although fig.3 indicates that peak I protease had activity in the absence of Ca^{2+}, as little as 100 μM EDTA could abolish this activity completely (data not shown). Therefore, it is likely that the protease activity observed in the absence of added Ca^{2+} in fig.3 was the result of trace amounts of Ca^{2+} or some other divalent cation in the reagents used in this experiment.

4. Discussion

Although there are reports of multiple forms of Ca^{2+}-dependent proteases, little has been done to characterize the isolated fractions. Three peaks of Ca^{2+}-dependent protease activity were found on ion-exchange chromatography of rabbit skeletal muscle extracts [6]. Only one of these protease activities removes muscle fiber Z-lines [6]. Major and minor peaks of Ca^{2+}-dependent protease were found on ion-exchange chromatography of rat brain extracts [2], but only the major activity peak (peak II) was studied. Two forms of Ca^{2+}-dependent protease from rabbit skeletal muscle were shown to differ in substrate specificity [11]. The two canine cardiac protease activities described here are clearly related. They appear to have the same molecular weight (fig.2), very similar pH-activity profiles and similar sensitivities to protease inhibitors. Peak I protease was a minor activity form of Ca^{2+}-dependent protease in the canine cardiac preparation described above. However, it has been observed that peak I protease in extracts from fresh rabbit skeletal muscle represents a significant fraction of total Ca^{2+}-dependent protease activity [11]. In some physiological conditions, peak I protease may perhaps be a major protease form in cardiac muscle. Studies to test this possibility are currently in progress.

It is significant within the context of this work that the two Ca^{2+}-dependent protease activities which have been purified to homogeneity from skeletal muscle [4,5] appear from their Ca^{2+} requirements to be peak II-type enzymes. Evidence that a peak I-type protease activity may be of paramount importance in the degradation of intracellular structural proteins is found in [8]. These investigators found that the protease in crude brain extracts which attacked microtubule-associated proteins and prevented microtubule assembly had half-maximum activity in the presence of ~50 μM Ca^{2+}. Since peak I protease is present in dog heart and rabbit skeletal muscle, it is reasonable to expect that this protease form is of key importance in the Ca^{2+}-dependent proteolysis of muscle cell microtubules and other ultrastructures.

Our current work is directed toward purification of the two canine cardiac proteases. These activities were unstable during further purification steps following the DEAE–Sephadex chromatography step. Hence, it was not possible to purify either protease form to homogeneity. The apparent low concentration of peak I protease in canine cardiac preparations further complicated attempts to purify this enzyme. Thus, it will probably be necessary to use large amounts of tissue to purify peak I protease to homogeneity.

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